

SECOND EDITION

Recombinant DNA



James D. Watson
Michael Gilman

Jan Witkowski
Mark Zoller

Recombinant DNA

SECOND EDITION

James D. Watson, Director, Cold Spring Harbor Laboratory
Michael Gilman, Senior Staff Investigator, Cold Spring Harbor Laboratory
Jan A. Witkowski, Director, Banbury Center, Cold Spring Harbor Laboratory
Mark J. Zoller, Senior Scientist, Genentech, Inc.

"Extraordinary" . . . "Masterful" . . . "Brilliant" . . . The first edition of *Recombinant DNA* inspired these enthusiastic responses and sold over 100,000 copies. Its uncomplicated language and exceptional diagrams combined to make the cutting edge of recombinant DNA technology supremely accessible to the uninitiated reader. Now the eagerly awaited *Second Edition of Recombinant DNA* takes its place as the authoritative introduction to the concepts and techniques of recombinant DNA research and their dramatic results.

Completely updated and revised by Nobel Laureate James Watson and his colleagues Michael Gilman, Jan Witkowski, and Mark Zoller, the new edition is extraordinary in its scope and clarity of information. To reflect the significant advances in molecular genetics and recombinant DNA technology since the first edition, the book features 14 chapters on new topics and 11 totally rewritten chapters, and incorporates research published throughout 1991.

As before, the coverage of *Recombinant DNA* is unique in centering largely on key experiments, with sections focusing on new developments in cloning, mutagenesis, and genetic engineering, plus the contribution of recombinant DNA technology to our understanding of gene function, biological processes, and human genetics. The illustrations—now in spectacular full color and nearly twice as numerous—offer self-explanatory portrayals of those experiments, conveying important concepts and delivering key information.

As an expert summary of the remarkable accomplishments of recombinant DNA technology, *Recombinant DNA, Second Edition* is an essential text for undergraduate, graduate, and professional courses in biology, genetics, biochemistry, cell biology, and molecular biology. With its detailed, lucid descriptions of human molecular genetics, the book is ideal for physicians at all stages of their careers, and its breadth of coverage makes it a valuable sourcebook for forensic scientists, patent attorneys, and everyone who needs to know how recombinant DNA is affecting our lives.

SCIENTIFIC AMERICAN BOOKS

Scientific American Books, Inc., is a subsidiary of
Scientific American, Inc.

Distributed by W. H. Freeman and Company
41 Madison Avenue
New York, New York 10010

20 Beaumont Street
Oxford OX1 2NQ, England
0-7167-2282-8

Cover Illustration by Marvin Mattelson

ISBN 0-7167-2282-8

RECOMBINANT DNA 2/92

000 100 000000 1 00 00

Foreign Genes Become Integrated in the Chromosomes of Recipient Animals

Before the advent of recombinant DNA techniques, the only sources of large amounts of pure genes were viruses. In the early studies designed to introduce DNA into mouse embryos, purified SV40 DNA was microinjected into the blastocoels of mouse embryos at the blastocyst stage. When the injected blastocysts were reimplanted into the uteri of foster mothers and allowed to develop, about 40 percent of the progeny had SV40 DNA in some of their cells. The mice were mosaic: in each individual tissue, some cells had SV40 DNA in their chromosomes and other cells did not. This proved that foreign DNA injected into very early embryos can be incorporated into the chromosomes of some embryonic cells and maintained in them as they both proliferate and differentiate into adult tissues. Similar experiments using the Moloney murine leukemia virus (MoMLV) showed that the provirus could become integrated into the germ line.

Once cloned genes became available, they could be microinjected into early mouse embryos. The best technique has proved to be microinjection of the cloned genes into fertilized eggs, which contain two pronuclei, one from the sperm (male), and one from the egg (female), that ultimately form the nucleus of the one-celled embryo. A few hundred copies of the foreign DNA in about 2 picoliters of solution are microinjected directly into one of the two pronuclei; the injected embryos are then transferred to the oviduct of a foster mother, and upon subsequent implantation in the uterus, many develop to term (Figure 14-1). The percentage of eggs that survive the manipulation and develop to term varies, but it is usually between 10 and 30 percent. Of the survivors, the number that have the foreign DNA integrated into their chromosomes is between a few percent and 40 percent. The introduced DNA appears to integrate randomly without preference for a particular chromosomal location, usually in a tandem array of many copies at a single locus. Mice that carry the foreign gene are referred to as *transgenic*, and the foreign DNA is termed a *transgene*.

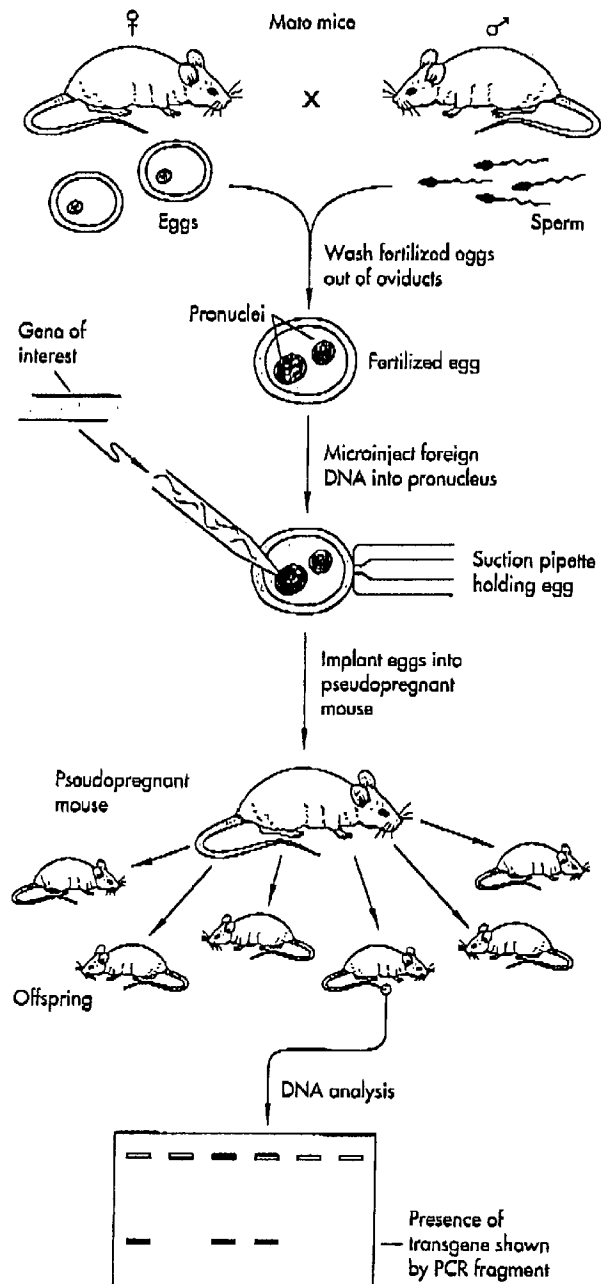


FIGURE 14-1

Producing transgenic mice by microinjection. Fertilized eggs are collected by washing out the oviducts of mated females, and the gene of interest is injected into one of the two pronuclei. The injected eggs are transferred to foster mothers, female mice made pseudopregnant by mating with vasectomized males. Three weeks after the birth, the offspring are checked for the presence of the transgene by Southern blotting of DNA extracted from a small piece of the tail. Screening can be performed rapidly using the polymerase chain reaction if suitable primers are available. In the example shown, three of the offspring carry the transgene.

Foreign DNA Can Become Stably Integrated into Germ Line Cells

It was quickly shown that DNA microinjected into one-celled embryos could be stably integrated in both somatic cells and germ line cells. Mice derived from embryos injected with cloned human interferon DNA or with rabbit β -globin DNA transmitted these transgenes to their offspring as a Mendelian trait just as with their own genes (Figure 14-2). All the mice derived from such a single founder mouse form a *line* of mice; every member of a line of mice has the same transgene at the identical position in its genome. The transmission pattern implies that the integration event occurs very early in development, before the first cell division of the zygote, and certainly before the germ cell population that gives rise to eggs or sperm is segregated from the primordial somatic cells. Once it was shown that foreign genes introduced into embryos are present in a stably integrated form in the somatic and germ cells of adult mice, it was necessary to determine whether these genes are expressed and, if so, whether this expression is appropriately regulated.

Embryonic Stem Cells Can Carry Foreign Genes

Direct injection of DNA into the pronuclei of fertilized mouse eggs is an efficient way of producing transgenic mice, but there is no opportunity to manipulate or otherwise control DNA integration. However, this can be done by introducing the DNA into special cells called *embryonic stem cells (ES cells)* and then injecting the transfected cells into embryos, where they become incorporated into the developing embryo (Figure 14-3). The ES cells are obtained by culturing the inner cell mass of mouse blastocysts. They are grown in tissue culture just like other cells except that the ES cells must be prevented from differentiating by growing them on a feeder layer of fibroblasts, or by adding leukemia inhibitory factor (LIF) to the culture medium. Under these conditions, ES cells can be grown for many weeks but still retain a remarkable capacity for differentiation—myocardium, blood vessels, myoblasts, cartilage, and nerve cells can be obtained. These extraordinary ES cells can be regarded as the equivalent of unicellular mice, and when they are injected into mouse blastocysts, they are able to participate in the formation of all tissues.

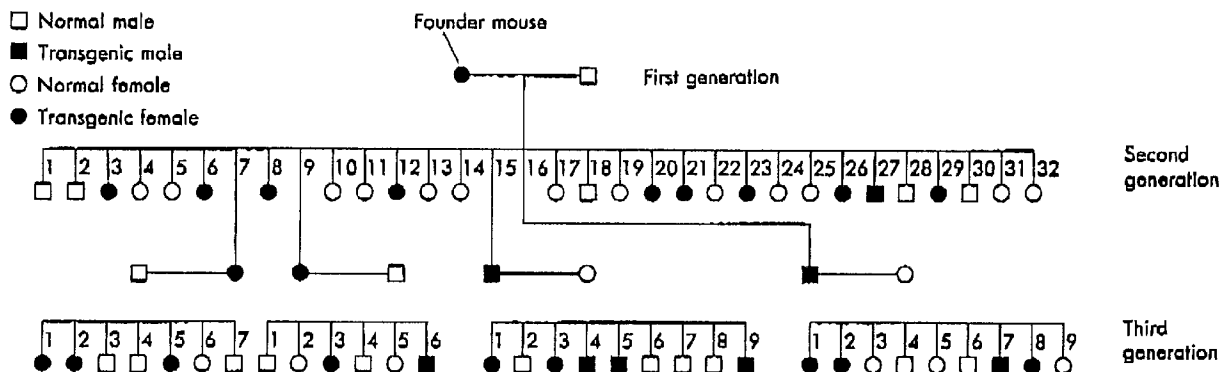


FIGURE 14-2

Microinjected foreign DNA can be transmitted through the germ line as with a normal Mendelian gene. It is important to determine whether the transgene has been integrated into the germ line as well as the somatic cells of transgenic mice because mice with the transgene integrated into germ line cells can be used to establish lines of transgenic mice. To do this, transgenic mice are mated with normal, nontransgenic mice and the inheritance of the transgene determined. In this case, a female mouse transgenic for the herpes simplex virus thymidine kinase gene linked to the promoter for the metallothionein gene was mated to a normal male. Approximately half the offspring in each generation carried the transgene, as would be expected for a transgenic mouse with a germ line integration.

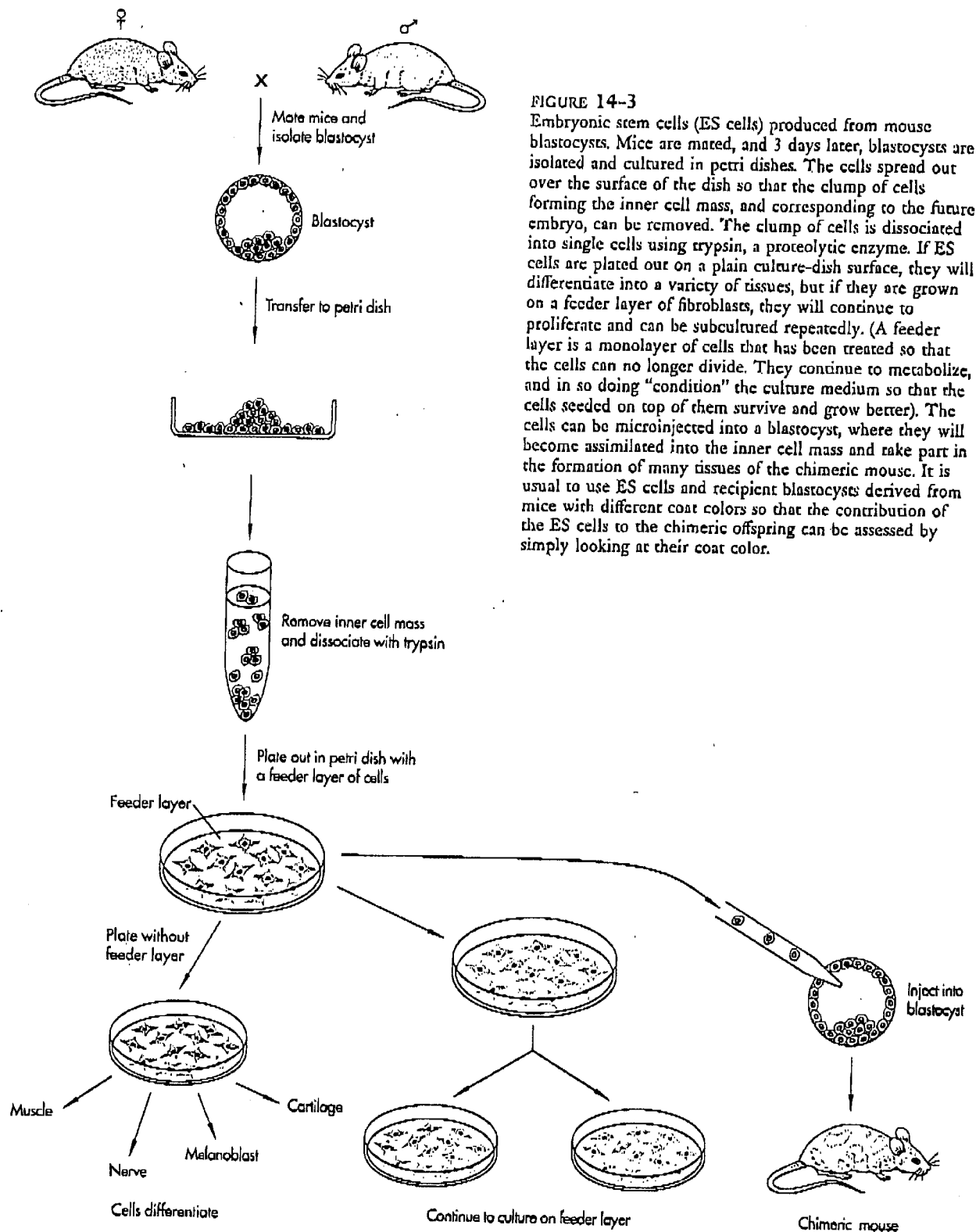


FIGURE 14-3

Embryonic stem cells (ES cells) produced from mouse blastocysts. Mice are mated, and 3 days later, blastocysts are isolated and cultured in petri dishes. The cells spread out over the surface of the dish so that the clump of cells forming the inner cell mass, and corresponding to the future embryo, can be removed. The clump of cells is dissociated into single cells using trypsin, a proteolytic enzyme. If ES cells are plated out on a plain culture-dish surface, they will differentiate into a variety of tissues, but if they are grown on a feeder layer of fibroblasts, they will continue to proliferate and can be subcultured repeatedly. (A feeder layer is a monolayer of cells that has been treated so that the cells can no longer divide. They continue to metabolize, and in so doing "condition" the culture medium so that the cells seeded on top of them survive and grow better). The cells can be microinjected into a blastocyst, where they will become assimilated into the inner cell mass and take part in the formation of many tissues of the chimeric mouse. It is usual to use ES cells and recipient blastocysts derived from mice with different coat colors so that the contribution of the ES cells to the chimeric offspring can be assessed by simply looking at their coat color.

DNA can be introduced into ES cells by transfection, retroviral infection, or electroporation (Chapter 10). Their most important advantage for gene transfer into mice is that cells carrying the transgene can be selected for before being injected into a blastocyst. In early experiments, ES cells were infected with retroviral vectors, or transfected with plasmids, carrying the *neo* gene. This gene confers resistance to the antibiotic G418. Only ES cells that have taken up the *neo* gene grow in medium containing G418, and these G418-resistant cells were introduced into mouse blastocysts. Not only did the resulting mice have *neo* integrated into their genomes, as shown by Southern blotting, but also the gene was transmitted to the offspring of the mice, and cell lines from the F2 generation were G418-resistant. Because ES cells can be manipulated in vitro before injection into the embryo, mouse geneticists can use homologous recombination to produce transgenic mice with mutations in specific genes, or to replace a mutant gene with the normal equivalent (Chapter 28).

Transgenes Can Be Regulated in a Tissue-Specific Pattern

Although a transgene integrates in a chromosomal location different from that of its endogenous counterpart, it is often expressed in a manner that mimics the expression of the endogenous gene. To determine the pattern of expression, various tissues are analyzed for the presence of RNA or protein products encoded by the transgene. Species differences may be capitalized on to distinguish the transgene product from its endogenous counterpart. For example, the RNA encoded by the human insulin gene can easily be differentiated from the RNA transcribed from the mouse insulin gene.

Insulin is a polypeptide hormone involved in the regulation of glycogen metabolism. This protein is normally produced in the β cells, which are found in discrete clusters of endocrine cells, called the *islets of Langerhans*, in the pancreas. When transgenic mice harboring the human insulin gene were analyzed, hu-

man insulin RNA was found in the pancreas but not in other tissues. Transcription of the human insulin transgene was induced by the same signals that induced the endogenous mouse insulin genes. Thus, not only can a foreign transgene be expressed in the correct tissue, but it may be subject to the same regulatory signals as the endogenous genes. Similar results have been obtained using many genes introduced into transgenic mice by ES cell transfer.

Transgene Expression Can Be Targeted to Specific Tissues

If the sequences responsible for tissue-specific regulation of a gene are known, they can be used to target expression of a gene product to a tissue in which it is not normally expressed. For example, the islets of Langerhans in the pancreas are composed of four cell types— α , β , δ , and PP—characterized by the hormone that they produce—glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. The promoter-enhancer control region of the insulin gene was used to target expression of SV40 large T antigen, a viral oncogene, to the β cells of the islets. The recombinant DNA molecule consisted of 660 bp from the 5' region of the rat insulin gene linked to the region of the SV40 genome encoding T antigen (Figure 14-4). Mice carrying this transgene died at 9 to 12 weeks of age, and pathological analysis showed hyperplasia (abnormal proliferation of cells) and tumors of the islets of Langerhans. All other tissues of the transgenic mice were normal despite containing the transgene, showing that tissue-specific expression was occurring in the islets. All the mice carrying this transgene developed pancreatic tumors, although only a minority of islets in each mouse was affected. Immunohistochemical analysis of tissue sections from the transgenic mice showed that the tumor cells expressed T antigen and that they were exclusively β cells. That is, the insulin gene control region had directed expression of the T antigen to precisely the appropriate cell type. These β -cell tumors are similar to a human tumor called an *insulinoma* except that the naturally occurring tumors also involve the δ cells of the islets.

Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER^T and Cre-ER^{T2} recombinases

Arup Kumar Indra, Xavier Warot, Jacques Brocard, Jean-Marc Bornert, Jia-Hao Xiao¹, Pierre Chambon* and Daniel Metzger

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France and ¹Allergan, 2525 Dupont Drive, Irvine, CA 92623-9534, USA

Received September 6, 1999; Accepted October 4, 1999

ABSTRACT

Conditional DNA excision between two LoxP sites can be achieved in the mouse using Cre-ER^T, a fusion protein between a mutated ligand binding domain of the human estrogen receptor (ER) and the Cre recombinase, the activity of which can be induced by 4-hydroxy-tamoxifen (OHT), but not natural ER ligands. We have recently characterized a new ligand-dependent recombinase, Cre-ER^{T2}, which was ~4-fold more efficiently induced by OHT than Cre-ER^T in cultured cells. In order to compare the *in vivo* efficiency of these two ligand-inducible recombinases to generate temporally-controlled somatic mutations, we have engineered transgenic mice expressing a LoxP-flanked (floxed) transgene reporter and either Cre-ER^T or Cre-ER^{T2} under the control of the bovine keratin 5 promoter that is specifically active in the epidermis basal cell layer. No background recombinase activity could be detected, while recombination was induced in basal keratinocytes upon OHT administration. Interestingly, a dose-response study showed that Cre-ER^{T2} was ~10-fold more sensitive to OHT induction than Cre-ER^T.

INTRODUCTION

The efficient introduction of somatic mutations in a given gene, at a given time, in a specific cell type, will greatly facilitate studies of gene function in vertebrates. We have previously established a temporally-controlled site-specific recombination system in mice using a conditional Cre/lox system. The fusion of the Cre recombinase with a mutated ligand binding domain (LBD) of the human estrogen receptor (ER) resulted in a tamoxifen-dependent Cre recombinase, Cre-ER^T, that was activated by 4-hydroxy-tamoxifen (OHT), but not by estradiol (1). In mice, the Cre-ER^T transgene placed under the control of a cytomegalovirus promoter was expressed in most organs analyzed, but not in all cell types. In the epidermis, Cre-ER^T was selectively expressed

in the granular cell layer (2). After a few days of tamoxifen treatment, Cre-ER^T was translocated from the cytoplasm to the nucleus, and shown to be active in essentially all cells of the granular layer (2), thus indicating that cell-specific expression of Cre-ER^T in transgenic mice could be used for the generation of site-specific somatic mutations in a spatio-temporally-controlled manner. Short-term tamoxifen treatments have low acute toxicity and cause no severe abnormalities in mice (3). However, to avoid possible undesired tamoxifen-induced side effects, we recently constructed additional Cre-ER mutants, and showed that, in F9 embryonal carcinoma cells, the OHT sensitivity of the Cre-ER^{T2} mutant containing the G400V/M543A/L544A triple mutation in the human ER LBD, was ~4-fold higher than that of Cre-ER^T (4).

Cells from the basal layer of the skin epidermis undergo a well-defined program of differentiation, coordinated with vertical migration to form suprabasal layers (spinous and granular layers) and a protective cornified layer of enucleated and keratinized cells, which is eventually shed. Thus, epidermal cells are continuously renewed throughout life, utilizing reservoirs of stem cells located in the basal layer and in the outer root sheaths of hair follicles (5). To create temporally-controlled somatic mutations in the epidermis, and to compare the efficiencies of Cre-ER^T and Cre-ER^{T2} *in vivo*, we have now established transgenic mice expressing these chimeric recombinases under the control of the bovine keratin 5 (K5) promoter which is selectively active in epidermal proliferating basal keratinocytes (6). For each chimeric Cre recombinase, we have selected transgenic lines that exhibit similar patterns and levels of expression. We show that excision of LoxP-flanked (floxed) DNA occurred in cells of the basal layer, upon OHT administration (1 mg/day for 5 days) to double transgenic mice that carry either one of the two chimeric recombinases together with the Cre recombinase reporter present in the ACZL transgenic line (7). Interestingly, in both cases no background activity could be detected in the absence of OHT administration. However, the OHT dose-response of Cre-ER^{T2} was ~10-fold more sensitive than that of Cre-ER^T for both nuclear translocation and recombinase activity. Thus, Cre-ER^{T2} may be preferred to generate spatio-temporally-controlled somatic mutations in the mouse,

*To whom correspondence should be addressed. Tel: +33 3 88 65 32 13; Fax: +33 3 88 65 32 03; Email: chambon@igbmc.u-strasbg.fr

particularly in embryos during the course of gestation. Furthermore, the present K5-Cre-ER^{T2}-expressing transgenic lines provide a tool to generate such mutations in the mouse epidermis.

MATERIALS AND METHODS

Generation of Cre-ER^T and Cre-ER^{T2} transgenic mice

pK5-Cre-ER^T and pK5-Cre-ER^{T2} plasmids were constructed as follows. The 0.85 kb *StuI*-*SalI* fragment, containing the rabbit β -globin intron II, a polycloning site and the SV40 polyadenylation signal, isolated from pSG5 (8), was inserted into the *StuI*-*XhoI* sites of pMCS-1 (9), resulting in pGS. The 2 kb *EcoRI* fragments isolated from pCre-ER^T (1) and pCre-ER^{T2} (4) were cloned into the *EcoRI* site of pGS, resulting in pGS-Cre-ER^T and pGS-Cre-ER^{T2}, respectively. The 5.2 kb *SalI* fragment, containing the bovine keratin K5 promoter and isolated from pK18-BK5P, was then cloned into the *SalI* site of pGS-Cre-ER^T and pGS-Cre-ER^{T2}, yielding pK5-Cre-ER^T and pK5-Cre-ER^{T2}, respectively. pK18-BK5P was constructed by cloning the 7 kb *KpnI* fragment isolated from pCKIII and containing the bovine keratin 5 gene promoter (10,11) into the *KpnI* site of pK18 (GenBank accession no. SYN8KMRCG). The 8 kb K5-Cre-ER^T and K5-Cre-ER^{T2} fragments were excised from pK5-Cre-ER^T and pK5-Cre-ER^{T2} plasmids by digestion with *NorI*, purified through a 10–30% sucrose gradient, and injected into C57BL/6 \times SJL F1 zygotes, as described (1). The Cre-ER^T and Cre-ER^{T2} transgenes were detected in mouse tail DNA by PCR using the primers TK139 (5'-ATTTGCCTGCATTACCGGTC-3') and TK141 (5'-ATCAACGTTTCTTTTCGG-3') located on the 5' and 3' side of the Cre gene, respectively (12). PCR detection of the ACZL transgene and generation of double transgenic mice were as described (2).

Preparation and administration of 4-hydroxy-tamoxifen (OHT)

Ethanol was added to 10 mg of OHT to obtain a 10 mg/100 μ l OHT suspension. A 10 mg/ml OHT solution was prepared by addition of autoclaved sunflower oil, followed by 30 min sonication with a Branson ultra-sonicator (Model 1210). Further dilutions were made in autoclaved sunflower oil and stored either at 4°C for a week or at -20°C for months. The OHT stock solutions were sonicated before use, and 100 μ l aliquots containing either 1, 0.1 or 0.01 mg OHT were injected intraperitoneally into 8–10 week old transgenic mice for 5 consecutive days as described (1).

Histochemistry

Immunohistochemistry was performed as described (2). For β -galactosidase histochemistry, 10 μ m-thick longitudinal sections were cut with a cryostat (Leica CM 3050), stained with X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactoside) as described (13), counterstained with 0.01% safranin, and dehydrated in ethanol (90 and 100%; 2 min each) and in LMR-SOL (Labo moderne, 2 \times 3 min) before mounting the cover-slips.

RESULTS AND DISCUSSION

For both Cre-ER^T and Cre-ER^{T2} transgenes, three transgenic founder animals were identified by PCR analysis of tail DNA. All founders were fertile and yielded transgenic lines. To characterize

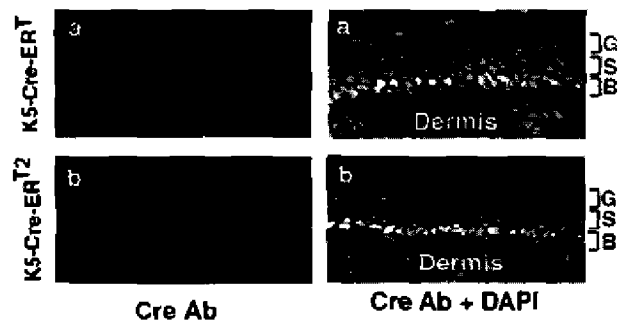


Figure 1. Expression of Cre-ER^T and Cre-ER^{T2} in tail epidermis of K5-Cre-ER^T and K5-Cre-ER^{T2} transgenic mice. Eight-week-old transgenic mice were injected for 5 consecutive days with OHT (1 mg/day). Immunohistochemistry with an antibody directed against Cre (Cre Ab) was performed on cryosections of tail biopsies of K5-Cre-ER^T (a, a') and K5-Cre-ER^{T2} (b, b') transgenic mice, taken 24 h after the last OHT injection. The red color corresponds to the staining of the chimeric recombinases (a, a' and b, b') and the cyan color to the DAPI staining of the nuclei (a', b'); the white color of the basal cell nuclei (a', b') results from the superimposition of the red color of the anti-Cre signal and the cyan color of the DAPI staining. B, S and G correspond to the basal, spinous and granular layers, respectively.

the expression pattern of the chimeric recombinases, immunohistochemistry analyses were performed on frozen skin sections, using an anti-Cre rabbit polyclonal antibody and confocal microscopy. Using DAPI to stain cell nuclei, basal cells were found to be specifically Cre-positive in the epidermis of all three K5-Cre-ER^T and K5-Cre-ER^{T2} lines (see Fig. 1 and data not shown). Two transgenic lines (one line for each chimeric Cre recombinase), exhibiting similar patterns and levels of Cre expression, were selected for further studies. Both Cre-ER^T and Cre-ER^{T2} proteins were essentially nuclear upon a 5 day treatment with 1 mg OHT/day (Fig. 1), in agreement with our previous results obtained with CMV-Cre-ER^T expressing mice in which Cre-ER^T was selectively expressed in the granular layer of the epidermis (2). In the absence of ligand treatment both Cre-ER^T and Cre-ER^{T2} proteins were located in the cytoplasm of basal cells, but at a lower level than in the case of transgenic mice expressing Cre-ER^T in the granular layer (Fig. 2 and data not shown; see ref. 2).

To estimate the binding of OHT to Cre-ER^T and Cre-ER^{T2}, we compared the intracellular localization of the chimeric proteins upon treatment of the transgenic mice with decreasing doses of OHT. Two days of 1 mg OHT treatment were sufficient to induce an efficient nuclear translocation of both Cre-ER^T and Cre-ER^{T2} proteins (Fig. 2, compare panels a and a' and b and b', with panels e and e' and f and f', respectively). In contrast, a 2 day 0.1 mg OHT treatment resulted in a stronger nuclear localization for Cre-ER^{T2} than for Cre-ER^T that was fully translocated into nuclei after 5 days of treatment only (Fig. 2, compare panels g and g' and panels c and c', respectively; and data not shown). A 2 day 0.01 mg OHT treatment was sufficient to translocate some Cre-ER^{T2} into nuclei (panels h and h'), while no Cre-ER^T positive nuclei could be observed (panels d and d'), even upon a 5 day treatment (data not shown), clearly indicating that lower doses of OHT were required to translocate Cre-ER^{T2} into basal cell nuclei.

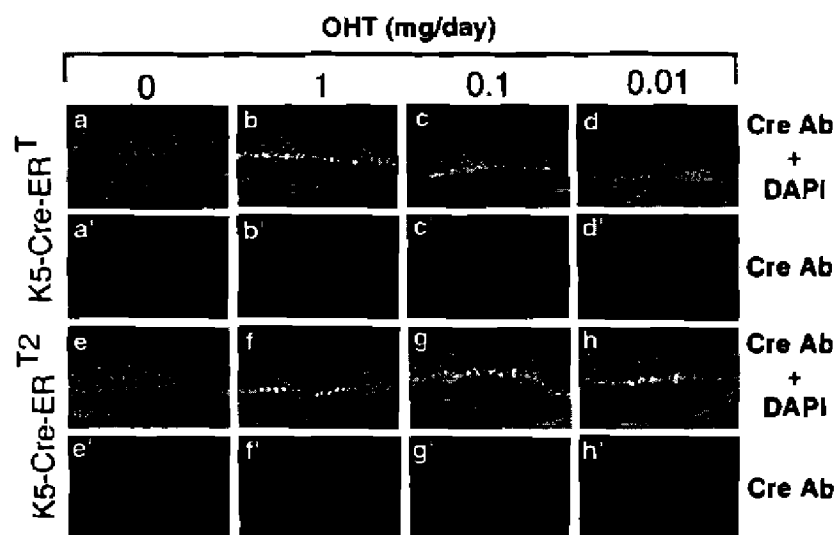


Figure 2. Comparison of OHT-induced nuclear translocation of K5-Cre-ER^T and K5-Cre-ER^{T2} proteins. Immunohistochemistry with Cre Ab was performed on cryosections of tail biopsies of 8-week-old K5-Cre-ER^T (a–d, a'–d') and K5-Cre-ER^{T2} (e–h, e'–h') double heterozygous transgenic mice. Mice were injected for 2 consecutive days with 1 mg/day (b, b', f, f'), 0.1 mg/day (c, c', g, g') and 0.01 mg/day (d, d', h, h') OHT. Tail biopsies were also taken before the first 1 mg OHT injection [(a, a', e, e')]. The cyan color (a–d and e–h) corresponds to the DAPI staining and the red color (a–d, a'–d' and e–h, e'–h') corresponds to the staining of the Cre-ER^T and Cre-ER^{T2}, respectively.

To compare the recombinase activity of the two chimeric recombinases, K5-Cre-ER^T and K5-Cre-ER^{T2} transgenic mice were crossed with the ACZL Cre recombinase reporter mouse line (7). In this line the expression of a loxP-chloramphenicol acetyl transferase (CAT) cassette-loxP-lacZ cassette is driven by the CMV enhancer and the chicken β -actin promoter (14), and β -galactosidase is produced only after Cre-mediated excision of the loxP-flanked (floxed) CAT cassette. However, due to cell-type restricted promoter activity (7 and data not shown), the Cre recombinase reporter transgene is not expressed in all cell types, and within the epidermis it is selectively expressed in keratinocytes of the granular layer (2 and data not shown). K5-Cre-ER^T/ACZL and K5-Cre-ER^{T2}/ACZL double heterozygote transgenic mice were examined for Cre-mediated excision upon 1, 0.1 and 0.01 mg OHT treatments. To this end, mice were injected for 5 days with OHT, and X-Gal staining was performed on tail skin sections 10 days after the last injection, in order to allow basal cells to differentiate into granular cells. Whereas no X-Gal staining could be detected in the skin of untreated or vehicle-treated K5-Cre-ER^T/ACZL and K5-Cre-ER^{T2}/ACZL mice, 1 mg OHT treatment resulted in both cases in blue staining of suprabasal cells (Fig. 3 and data not shown). Similar blue stainings were observed 25 days after the last OHT injection (data not shown). As suprabasal cells are renewed in 7–10 days in the mouse tail epidermis (2 and data not shown), these results show that recombination had occurred in proliferative basal cells, whereas the lack of blue staining in the absence of ligand treatment clearly indicates that the recombinase activities of both Cre-ER^T and Cre-ER^{T2} are fully dependent on OHT binding. Interestingly, the X-Gal staining pattern of the epidermis of K5-Cre-ER^{T2} mice was very similar upon a 0.1 mg OHT treatment, while upon the

same treatment not more than 30% of the suprabasal cells of K5-Cre-ER^T mouse epidermis was stained (Fig. 3 and data not shown). When 0.01 mg OHT was used, no X-Gal staining could be observed in K5-Cre-ER^T epidermis, whereas in the case of K5-Cre-ER^{T2} mice the staining was similar to that seen in 0.1 mg OHT-treated K5-Cre-ER^T mice (Fig. 3 and data not shown).

Taken all together, our results show that the recombinase activity of Cre-ER^{T2} can be induced in the mouse with OHT doses that are ~10-fold lower than those required to activate Cre-ER^T. Upon oral or topical tamoxifen administration, Vasioukhin *et al.* (15) recently reported an efficient excision of loxP-flanked sequences in epidermis basal cells of adult mice expressing another Cre-ER fusion protein, Cre-ERtm, under the control of the human K14 keratin promoter. Cre-ERtm is similar to Cre-ER^T, but contains the mouse, instead of the human, mutated ER LBD. Danielian *et al.* (16) have reported that the dose of ligand required to fully activate Cre-ERtm (another Cre-ER^T-like tamoxifen-inducible Cre recombinase) in developing mouse embryos is close to that which interferes with the maintenance of pregnancy. Thus, the present conditional Cre-ER^{T2} recombinase should be preferentially used to generate spatio-temporally controlled somatic mutations in the mouse, whenever tamoxifen administration may result in undesirable side effects, notably during gestation.

ACKNOWLEDGEMENTS

We are grateful to M. Blessing for the bovine K5 promoter and to A. Berns and M. Giovannini for ACZL mice. We thank S. Bronner, C. Gérard and N. Messaddeq for excellent technical help, M. LeMeur and the animal facility staff for animal care, the secretarial staff for typing the manuscript and the illustration

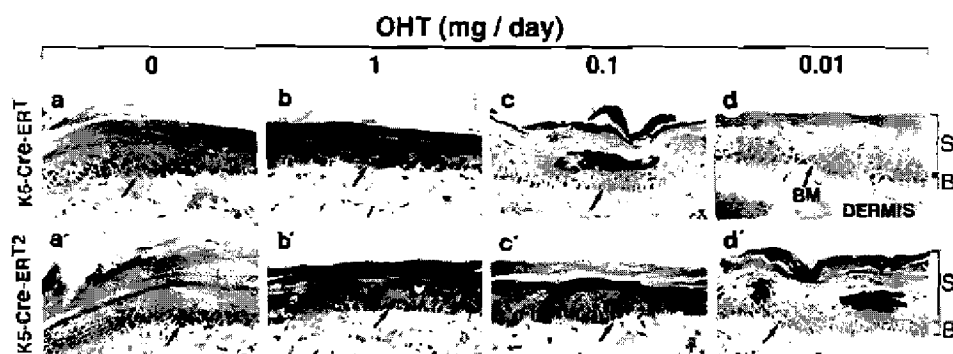


Figure 3. Comparison of the OHT-induced expression of β -galactosidase in tail epidermis of K5-Cre-ERT¹/ACZL (a–d) and K5-Cre-ERT²/ACZL (a'–d') double heterozygous transgenic mice. Eight-week-old K5-Cre-ERT¹/ACZL (a–d) and K5-Cre-ERT²/ACZL (a'–d') double heterozygous mice were injected daily with 1, 0.1 and 0.01 mg/day OHT from day 0 to 4. Tail biopsies collected just before the first OHT injection (0; a, a') or at day 15 following 1 mg (b, b'), 0.1 mg (c, c') and 0.01 mg (d, d') OHT injection, were stained with X-Gal (Materials and Methods). Arrows point to the basement membrane. B and S correspond to the basal and suprabasal layers, respectively.

staff for preparing the figures. This work was supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Collège de France, the Hôpital Universitaire de Strasbourg, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Human Frontier Science Program, the European Economic Community contract FAIR-CT97-3220 and the Ministère de l'Éducation Nationale de la Recherche et de la Technologie Déc.97.C.0115. A.K.I. was supported by a fellowship from the Université Louis Pasteur (Strasbourg), and J.B. and X.W. by fellowships from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie and from the Fondation pour la Recherche Médicale.

REFERENCES

- Feil, R., Brocard, J., Mascres, B., LeMeur, M., Metzger, D. and Chambon, P. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 10887–10890.
- Brocard, J., Warot, X., Wendling, O., Messaddeq, N., Vonesch, J.L., Chambon, P. and Metzger, D. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 14559–14563.
- Furr, B.J.A. and Jordan, V.C. (1984) *Pharmac. Ther.*, **25**, 127–205.
- Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997) *Biochem. Biophys. Res. Commun.*, **237**, 752–757.
- Millar, S. (1997) In Cowin, P. and Klymkowsky, M.W. (eds), *Cytoskeletal-Membrane Interactions and Signal Transduction*. Landes Bioscience, Austin, TX, pp. 87–102.
- Ramirez, A., Bravo, A., Jorcano, J.L. and Vidal, M. (1994) *Differentiation*, **58**, 53–64.
- Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M. and Berns, A. (1997) *Nucleic Acids Res.*, **25**, 1766–1773.
- Green, S., Issemann, I. and Sheer, E. (1988) *Nucleic Acids Res.*, **16**, 369.
- Feng, X., Peng, Z.H., Di, W., Li, X.Y., Rochette-Egly, C., Chambon, P., Voorhees, J.J. and Xiao, J.H. (1997) *Genes Dev.*, **11**, 59–71.
- Blessing, M., Nanney, L.B., King, L.E., Jones, C.M. and Hogan, B.L.M. (1993) *Genes Dev.*, **7**, 204–215.
- Amendt, C., Schirmacher, O., Weber, H. and Blessing, M. (1998) *Oncogene*, **17**, 25–34.
- Sternberg, N., Sauer, B., Hoess, R. and Abremski, K. (1986) *J. Mol. Biol.*, **187**, 197–212.
- Takahashi, K. and Coulombe, P.A. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 14776–14781.
- Araki, K., Araki, M., Miyazaki, J. and Vassalli, P. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 160–164.
- Vasioukhin, V., Degenstein, L., Wise, B. and Fuchs, E. (1999) *Proc. Natl Acad. Sci. USA*, **96**, 8551–8556.
- Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K. and McMahon, A.P. (1998) *Curr. Biol.*, **8**, 1323–1326.